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Facilitating the indirect detection of genomic DNA in an electrochemical DNA biosensor using magnetic nanoparticles and DNA ligase

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ABSTRACT

A common problem in applying biosensors for the detection of genomic DNA is detecting short sequences in large amounts of long double stranded DNA. A gold electrode modified with a conductive nanocomposite, poly(3,4-ethylene-dioxythiophene), and gold nanoparticles was functionalized with 2,6-Pyridinedicarboxylic acid. Immobilization of a 20-mer DNA probe as the bioreceptor was successfully carried out via a peptide bond on the surface of the modified electrode. Two segments of 15 and 20 base probes were designed and named as Capture and Reporter probes respectively. The 20-mer Reporter probe was complementary to the bioreceptor and the 15-mer Capture probe was designed to bind on to the surface of the iron oxide magnetic nanoparticles. A 35-base Target DNA complementary to the Capture and the Reporter probes was used as Template in the ligation process, with the ligation between the Reporter and Capture probes mediated by T4 ligase. Iron oxide magnetic nanoparticles functionalized with carboxylic groups on their surface synthesized in a new method were attached to the 15-mer Capture probe. After the denaturation of the final ligation product, the separation of the attached probes was carried out using 5 G permanent magnets in a three step washing procedure in TE buffer. The hybridization of the DNA bioreceptor and the Reporter probe attached to the Capture probe-Fe_3O_4 was monitored via oxidation and reduction of the new redox marker (ruthenium complex) intercalated into the double helix.

This technique was found to be reliably repeatable. The indirect detection of genomic DNA using this method is significantly improved and showed high efficiency in small amounts of samples with the detection limit of 5.37×10^{-14} M.

1. Introduction

The design of the electrochemical biosensor described in this paper could equally have been applied to any genomic DNA but the white rot fungus, *Ganoderma boninense* was selected in this study. This is a pathogen, which causes both basal and upper stem rot in oil palms and presents a major threat to a highly lucrative industry in the south East Asia. *G. boninense* has a devastating effect on a plantation; it causes direct loss of the stands and reduces the yield of the palms, creating the need for earlier replanting [1]. As soon as young palms show symptoms of the disease they inevitably die within a year or two while older trees survive only a few years longer [2].

As well as trying a multitude of methods of control, extensive work has already been carried out on the early detection of *G. boninense* [3]. After experimenting with PCR [4], researchers

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tried polyclonal antibody for ELISA technique [5]. Finally, a multiplex PCR-DNA kit [6] was designed for the Malaysian Palm Oil Board although it is far from offering a complete solution. The PCR technique is sensitive to contamination, and because it can be inhibited by secondary metabolites, it is critical to purify the DNA before the reaction [7,8].

In the belief that DNA-based nanosensors and DNA microarrays should be easier to operate, faster, more accurate and more economically viable than PCR-based techniques, an electrochemical DNA biosensor was designed for detection of *G. boninense* [9,10]. This was developed based on a gold electrode modified with a nanocomposite membrane of poly(3,4-ethylenedioxy thiophene)-poly(styrenesulfonate) containing gold nanoparticles. While the sensor showed successful detection of *G. boninense*, the preparation of representative samples and the extraction of DNA remained a challenge and invited further study.

The research in this paper describes how Au nanoparticles were used to enhance the already constructed electrochemical DNA biosensor, magnetic nanoparticle in the separation step and the enzyme, ligase, to enable indirect detection.

Electrochemical biosensors work by producing an electrical signal that relates to the concentration of the biological analyte [11]. The relatively low cost and rapid response of DNA biosensors, in particular, promise exciting potential because of their simplicity, speed, and economical assays for gene analysis and testing.

In the construction of DNA biosensors, conducting polymers are effective platforms for the immobilization of biomolecules on electrode surfaces [12], providing good signal transduction, sensitivity, selectivity, durability, biocompatibility, direct electrochemical synthesis, and flexibility for the immobilization of DNA [13]. poly(3,4-ethyllenedioxythiophen)–poly(styrenesulfonate) (PEDOT-PSS) has shown good potential due to its function (Φ) ~5 eV [14]. It needs to be combined with poly(styrenesulfonate) (PSS) to enable its dispersion in water for forming thin films on surfaces [15].

Additionally, the explosion of nanotechnology and the use of nanomaterials in DNA biosensors is further enhancing immobilization and interface capabilities of the DNA material with the electrode and, ultimately, the detection signal response [16,17]. Metal nanoparticles are frequently added to conjugate polymers to boost the conductivity of the surface [15,18,19] and to increase the active surface area of the sensor, ultimately enhancing the transduction signal response [20–22]. In this work, gold (Au) was used.

Magnetic nanoparticles (MNPs), particularly iron oxide Fe_3O_4, are especially popular because of their powerful magnetic properties, large surface areas and the ease in which they can be separated from a liquid with a magnet [23].

Finally, as well as using gold and magnetic nanoparticles in our design, a novel addition was the use of the enzyme, ligase. DNA ligases have become indispensable tools in modern molecular biology research for generating recombinant DNA sequences - they recombine fragments of DNA from different sources into a new DNA molecule strand. Joining linear DNA fragments together with covalent bonds is called ligation and this involves creating a phosphodiester bond between the 3′ hydroxyl of one nucleotide and the 5′ phosphate of another.

The specific enzyme used to ligate DNA fragments here is T4 DNA ligase, which originates from the T4 bacteriophage. This enzyme will ligate DNA fragments with overhanging, cohesive ends similar to repairing 'nicks' in duplex DNA. T4 DNA ligase will also ligate fragments with blunt ends, although higher concentrations may be needed to do this.

As well as water, a ligation reaction requires three ingredients: two or more fragments of DNA that have either blunt or compatible cohesive ('sticky') ends, a buffer which contains ATP (Adenosine triphosphate). The buffer is usually provided or prepared as a 10X concentrate which, after dilution, yields an ATP concentration of roughly 0.25–1 mM. Most restriction enzyme buffers will work if supplemented with ATP and the T4 DNA ligase. Compared with PCR, the ligase detection reaction (LDR) provides better sequence specificity, even for single base variations.

The goal of the improvement to the sensitivity of the biosensor described in this paper, was to detect small sequences in large amounts of double stranded DNA, a desirable objective which had not been successfully achieved earlier.

2. Materials and methods

2.1. Reagents and solutions

The ruthenium complex $[Ru(dppz)_2(qtpy)Cl_2$; dppz = dipyrido [3, 2–a:2′, 3′-c] phenzine; dpty = 2, 2′, 4, 4′′. 4′, 4′′′–quaterpyridyl] was synthesized according to a previously documented method [24]. A stock solution of the ruthenium complex $[Ru(dppz)_2(qtpy)]Cl_2$ = Ru(dppz) was mixed with 50.0 mM trisaminomethane-HCl, 20.0 mM NaCl and methanol. Next, the stock solution was used to prepare a more dilute solution of 25 μM. Deionized water from a Milli-Q purifier was used in preparation of the supporting electrolyte and washing buffer solution of 10.0 mM trisaminomethane-HCl in 1 mM EDTA (TE) (pH 8.0), followed by the preparation of an ethanolic solution of 3.0 mM 3,3′-dithiopropionic acid (DPA).

An activation solution of 5.0 mM N–hydroxysulfosuccinimide in 2.0 mM 1-ethyl-3-[3-dimethylaminopropyl] carbo-diimidehydro chloride containing 50.0 mM sodium-phosphate was also prepared. The 5′ end of 20-mers probe DNA was modified with an amino (NH_2) group (5′–NH_2–C_6-CCT GCT GCG TTC TTCTTC AT-3′) and named as the Reporter probe. A 20-mers complementary Target probe (5′-ATG AAG AAG AAC GCA GCA GG-3′) and a 15-mers Capture probe (5′–NH_2–TTG GCT CTC GCA TCG-3′) to bind with the MNPs were synthesized by First Based Laboratories Sdn Bhd, Selangor, Malaysia. The Template for ligation was a 35-base DNA sequence (5′-CCT GCT GCG TTC TTCTTC ATC GAT GCG AGA GCC AA-3′) which had been carefully selected from *G. boninense*. This followed earlier work which has been reported more fully [3]. As alternatives, a noncomplementary DNA sequence (5′-GGA AGG CCA GCT ACA ACC CAG CTA GTC AAG GTA AC-3′), a single mismatch DNA Template (5′-CCT GCT GCG TṪC TTC TTC ATC GAT GCG AGA GCC AA-3′) and extracted genomic DNA from *G. boninense* were tested as Templates.

The stock solutions of 100 μM of the DNA molecules were prepared upon arrival and other dilute concentrations of 1×10^{-7} M to 3.13×10^{-9} M of the complementary Template DNA were prepared in trisaminomethane-HCl containing EDTA buffer solution (pH 8.0) and kept in a freezer. We defrosted the DNA solution as needed. All other reagents used are of analytical grade.

2.2. Apparatus and electrode

The equipment used to obtain voltammetry measurements was an AUTOLAB (Eco Chemie, The Netherlands) potentiostat linked to General-Purpose Electrochemical System (GPES 4.9, Eco Chemie) software. The electrochemical cell comprised a three-electrode system of Metrohm gold electrode (AuE) as the working electrode, platinum (Pt) wire as the counter electrode and also Ag/AgCl/KCl 3M as the reference electrode.

2.3. Synthesis of Au and Fe_3O_4nanoparticles

Synthesis of the gold nanoparticles was based on earlier reported processes [25–27] in which AuNPs were prepared by the

sodium citrate reduction of an $HAuCl_4.3H_2O$ solution.

The magnetic nanoparticles (MNPs) of Fe_3O_4 were synthesized, again based on an already tried method [28]. The method used here followed that described in the literature [29] with some slight modifications. Co-precipitation of Fe_3O_4MNPs occurred when $FeSO_4 4H_2O$ was stirred rapidly into a mixture of NaOH, $NaNO_3$ and $C_6H_5Na_3O_7 2H_2O$.

After cooling we used a 5 G permanent magnet to separate the synthesized MNPs from the solvent and washed these several times. The size of these particles was observed with TEM and their morphology by FESEM.

2.4. Modification of the electrode

The bare gold electrode was pretreated for modification by polishing it with an alumina slurry followed by 5 min of sonication in deionized water. The electrode was then dried under nitrogen gas, immersed in a concentrated sulfuric acid solution, again sonicated before rinsing with TE buffer. It was dried under gentle flow of nitrogen gas for 30 s and further dried at room temperature for 45 min.

Subsequently the dried pretreated gold electrode surface was drop coated with the prescribed volume of the PEDOT-PSS and cured for 15 h at 70 °C in oven. It was then rinsed with washing buffer to remove the unbounded PEDOT-PSS from the surface of the modified film of the PEDOT-PSS on the gold electrode surface.

Electrochemical investigations were performed using cyclic voltammetry (CV) in the TE supporting electrolyte both with the Ru(dppz) complex solution as well as without it. We also performed an additional modification, which was to immerse the modified electrode in a colloidal gold nanoparticles solution for 12 h. The washing and drying protocols used in this process were the same as previously explained. This additional step gave us a modified gold electrode/PEDOT-PSS/gold nanoparticle for voltammetry measurements.

2.5. Immobilization of probes

The process used to modify the AuE/PEDOT-PSS/AuNPs was to immerse it in an ethanolic solution of 3.0 mM 3,3′-dithiopropionic acid (DPA) for 45 min to form a monolayer on the electrode surface [30]. The unbound DPA molecules were removed by washing with deionized water and TE as washing buffer. The carboxylic group of the DPA was activated, the electrode was submerged for an hour at room temperature in a 5.0 mM N–hydroxysulfosuccinimide of 2.0 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide containing 50.0 mM phosphate solution. This creates the conditions needed for easy bonding with amine group attached to the *G. boninense* specific-sequence probe single stranded DNA.

That probe DNA was subsequently accumulated for immobilization on the 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide activated modified electrode for 2 h, resulting in the probe modified electrode, which was labeled AuE/PEDOT-PSS/AuNPs/ssDNA. The CV of the ssDNA probe modified electrode in TE buffer at scan rate of 100 mV/s using ruthenium Ru(dppz) redox indicator was performed.

2.6. Cultivation of the fungus and extraction of genomic DNA

The fungus was cultivated in potato dextrose broth and the mycelia were used for DNA extraction. Total DNA was extracted from the fungus mycelia, using the DNeasyPlant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions but with some slight modifications.

2.7. Ligation procedure

As per the SIGMA Kit instructions, we took the reagents from the freezer and thawed them on an ice bath to a temperature of −20 °C. We determined the ratio of components to achieve maximum ligation efficiency. The concentration of the limiting factor in our procedure was kept less than a tenth of the other probes. The limiting factor in selectivity test was the Target probe and in sensitivity test was the complementary Template DNA. We mixed this solution well and left it to incubate for 12–16 h at 16 °C. Subsequently we kept it on ice until we needed to use it.

2.8. Hybridization of DNA

The first step in the design of a DNA bisosensor is the hybridization of a Target DNA to its complementary sequence immobilized on the surface of biorecognition site. The DNA hybridization step in this research followed a protocol [31] in which a buffer solution containing 100 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$ and 3 mM $MgCl_2$ with pH 8.0 was used.

We studied the hybridized electrode (AuE/PEDOT-PSS/AuNPs/ssDNA:ssDNA) using cyclic voltammetry in Ru(dppz) complex solution as intercalating redox marker. This was the protocol also applied to the probe-modified electrode when we tested the hybridization reaction of a single-mismatched sequence of the Target DNA. We used a range of concentrations of Template DNA, ranging from 1×10^{-7} M to 3.13×10^{-9} M in TE buffer (pH 8.0). We also used this as a suitable concentration range for the passive hybridization or storage of DNA [32–35]. We performed the differential pulsed voltammetry (DPV) electrochemical measurements from scanning potential of 0.1 V–2.0 V at the scan rate of 100 mV/s in the presence of Ru(dppz).

3. Results and discussion

3.1. Principle of the procedure

Fig. 1 is a schematic diagram of the whole detection procedure. It depicts chemically synthesized magnetic nanoparticles functionalized with carboxylic groups and bound with 15-mers Capture probes.

The first step in this method is the hybridization of the Capture probe and its complementary region in the pre-denatured extracted genomic DNA and the 20-mers Target probe with its complementary sequence.

The ligation step is the second step in which T4 DNA ligase is employed to join the two probes (Capture and Target) together using ATP. The result of this step is a long 35-mer single stranded DNA bound to magnetic nanoparticles. The presence of two probes and their complementary region is crucial for this step as well as ATP and ligase because the enzyme is unable to bind two segments if they are not hybridized to their complementary region and therefore close enough for the reaction.

The third step is called ‘separation’. In this step, the complementary regions are separated by heating and magnetic nanoparticles are separated from the reaction by employing a magnetic field. In this step, if ligation has been done properly, what remains are magnetic nanoparticles bound to 35-mers single stranded DNA. If any of the main components of the ligation process are missing, particularly if the complementary region (Fig. 1b) is absent, then the remaining particles would only be bound to the 15-mers Capture probes.

The last step is the detection step. A modified gold electrode with gold nanocomposite was used to immobilize the 20-mers Reporter probe of DNA, complementary to the Target probe. The

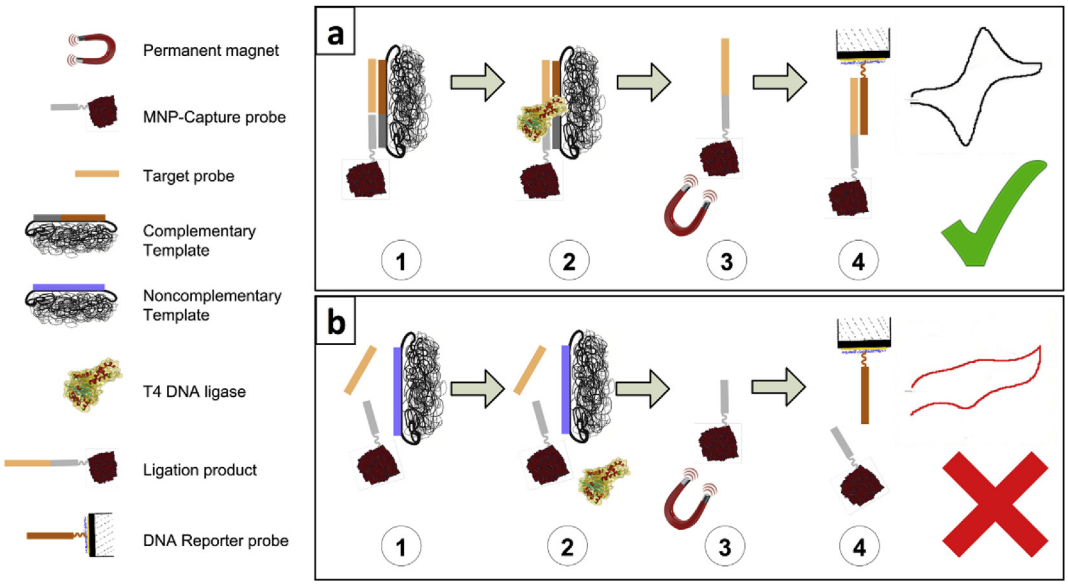

Fig. 1. Schematic diagram of the proposed method of detection. The stages are numbered in the figure: 1-Hybridization 2-Ligation 3- Separation 4-Detection a) at the presence of the complementary region in the genome. b) when the Target sequence doesn't exist in the sample.

presence of the Target probe was investigated with CV and DPV.

Fig. 2 illustrates the major components involved in the attachment of DNA probes to the modified electrode. Gold nanoparticles were used to increase the active surface area of the electrode. The immobilization of these particles was mediated by a conductive polymer called PEDOT. The surface of gold nanoparticles was then modified with DPA. DPA was used as a linker to immobilize the Reporter probe. The presence of Ru(dppz) was studied, using CV and DPV, taking into account that this complex has higher affinity for double helical DNA molecule than a single stranded one. Magnetic nanoparticles were used for the separation step only as they have no impact on the electrochemical reaction.

3.2. Characterization of the components by EM

Field emission scanning electron microscopy (FESEM) was employed to study the morphology and Transmission electron microscopy (TEM) was employed to study the size of the synthesized nanoparticles and the success in surface modification of the electrode. Fig. 3a is the TEM micrograph of the Au nanoparticles with dimensions of between 35 nm and 65 nm. The observed micrograph under FESEM (Fig. 3b) reveals the monodispersion of particles which are mostly spherical but also include some triangular and hexagonal plates.

An FESEM micrograph of the gold electrode modified by PEDOT-PSS (Fig. 3c.) and subsequently covered by AuNPs (Fig. 3d) shows that modification of the electrode was performed successfully prior to immobilization of the Reporter DNA probe. The AuNPs were used in this study to improve the active surface area of the electrode.

The TEM micrograph of the chemically synthesized MNPs (Fig. 3e.) shows the average size of 25 nm with a size distribution of between 10 nm and 45 nm. The cubic shape of the particles was apparent using FESEM (Fig. 3f).

3.3. Characterization of the electrode modification by CV and DPV

In an electrochemical DNA biosensor, the hybridization is assessed on the surface of the electrode. Prior to the immobilization of the DNA probe, the surface of the gold electrode used in this experiment was modified by a nanocomposite of conducting

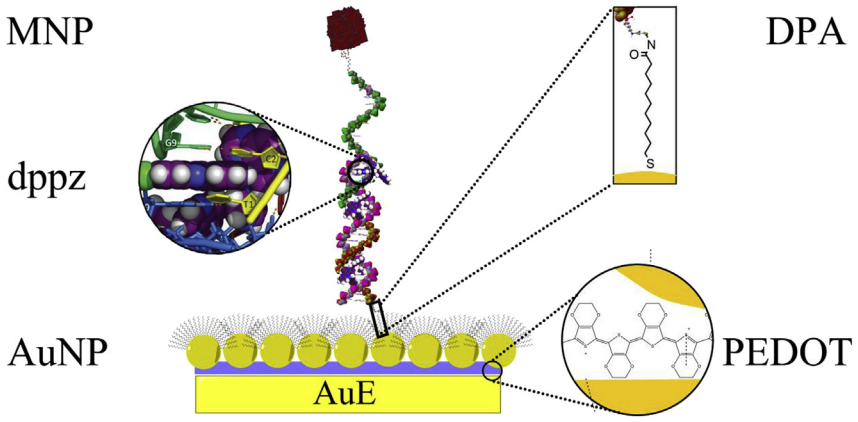

Fig. 2. Schematic of the major components of the designed electrochemical DNA biosensor. Showing the role of AuNPs to increase the surface area, detection with redox complex and the use of MNPs for separation.

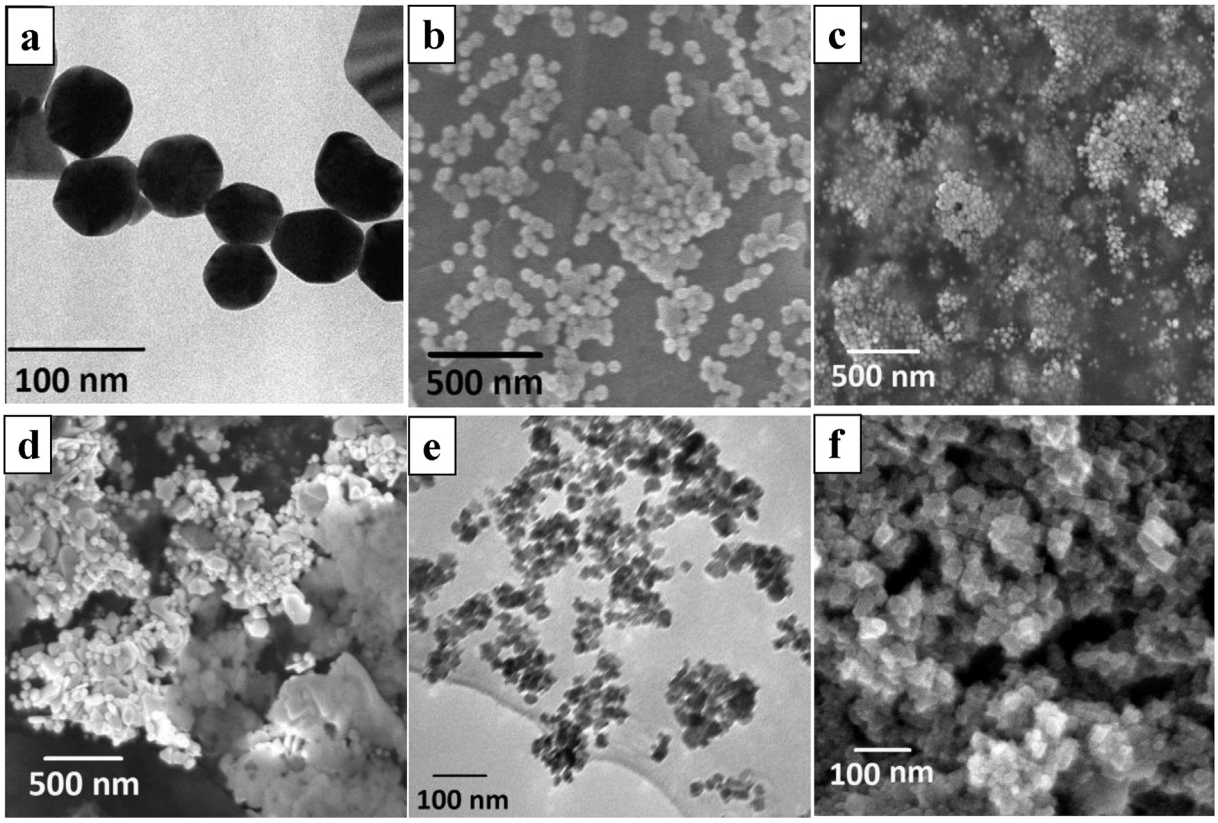

Fig. 3. Electron micrograph of the particles. a) TEM of the AuNPs. b)FESEM of the AuNPs. c) PEDOT-PSS on the surface of a gold electrode. d) modified gold electrode with PEDOT-PSS and AuNPs. e)TEM micrograph of Fe3O4nanoparticles. f)FESEM of the MNPs.

polymer of PEDOT-PSS and gold nanoparticles.

The modification of the electrode was investigated by FESEM in Fig. 3c and d as well as cyclic voltammetry (CV) (Fig. 4a) and differential pulse voltammetery (DPV) (Fig. 4b).

Both the CV and DPV study of the modification steps prove the success of the process by increasing the conductivity of the electrode. Additionally the conductive nanocomposite had a beneficial effect by increasing the active surface area of the electrode. Also interesting to note is that the DNA, in addition to having a negative charged backbone, because of the interaction with the redox complex increased the current further, after immobilization.

3.4. Evaluation of different samples

In our designed procedure T4 DNA ligase as well as the reaction buffer and ATP requires 2 segments of single stranded DNA successfully hybridized each with their complementary strand while they are butted up against each other.

Different ligation reactions were prepared, all with the same conditions and proportions but for the difference in complementary sequences. In three different reactions, one contained a synthesized Template exactly complementary to our probes, one had a single mismatch and one called noncomplementary DNA had a totally different sequence of bases. Our blank reaction had no Template and in the graphs depicted in Fig. 5, this is addressed as TE for Tris—HCl, EDTA. Extracted genomic DNA was used as the ligation Template in another reaction but here DNA was denatured at 94 $^{\circ}$C for 10 min since it was the only Template that was double stranded. A concentration of 10^{-7} M for the different Templates was used in this experiment. The concentration of the extracted genomic DNA

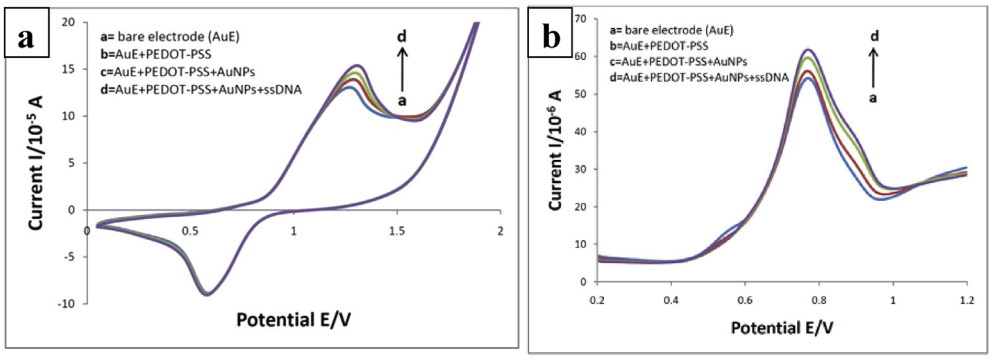

Fig. 4. Evaluation of the Electrode modification by a) cyclic voltammetry (CV) b) differential pulse voltammetry (DPV).

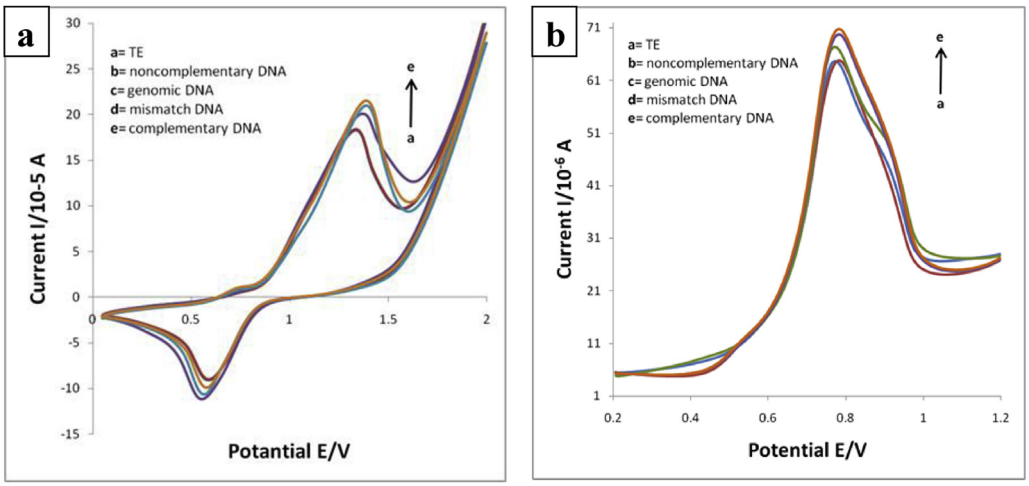

Fig. 5. Evaluation of the ligation reaction products of different Templates with a) cyclic voltammetry (CV) b) differential pulse voltammetry (DPV).

was calculated as 20 ng/μl. Since the genomic information of the tested pathogen is insufficient [3], the precise amount of the complementary sequence in the sample could not be identified. In addition, the DNA yield of the genomic extraction varies in relation to several different factors including genome size and the age of the samples. Thus, to be able to assess the genomic sample, a larger amount of the extracted DNA was used compared with other known synthesized sequences.

It is important to bear in mind that in this mechanism, the 20-base sequence Target probe is what we are trying to detect so we can take this sequence as the limiting factor in our litigation reaction. This is illustrated in Fig. 6.

The proportion of the three DNA segments – Capture, Template and Target – must always be 1:1:1 Capture and Target bind together at the presence of Template so the final product will be the concentration of that bind plus the unchanged Template.

If, however, one of the concentrations becomes less than the others, then any surplus becomes redundant in the final product which would be the total of the three concentrations without the two surplus amounts.

Thus, holding the Target concentration at 10^{-7} M allowed us to increase the quantity of genomic DNA and compare results from other samples.

By adjusting the initial concentrations:

$$\text{Capture}_1 > \text{Target}_1 \text{ and Template} > \text{Target}_1$$

The concentration of the Target probe before the reaction was the lowest of the components in the following reaction:

$$\text{Capture}_1 + \text{Target}_1 \rightarrow \text{Capture}_2 + \text{Target}_2 + \text{Product}$$

At the end of the reaction, the concentration of the Template DNA remained constant, the Target probe becomes the ligation Product and the extra Capture probes remain untouched.

Therefore:

$$\text{Capture}_2 = \text{Capture}_1 - \text{Target}_1$$

and

$$\text{Target}_2 = \text{Target}_1 - \text{Target}_1 = 0$$

The concentration of the ligation Product is equal to the concentration of the limiting factor, in this case, the Target probe:

$$\text{Product} = \text{Target}_1$$

After the final dehybridization and separation of the ligation reaction with a magnet, the resulting samples were studied in an electrochemical study. The lowest peaks, as expected, belonged to the ligation samples where no Template DNA was used and that was exactly the same result with the sample in which the Template DNA was noncomplementary. In both cases the ligation between Capture probe and Target probe did not occur so the Target probes washed out during the separation process. In CV (Fig. 5a) these two samples are not differentiated whereas in DPV (Fig. 5b) the two graphs are distinguished yet having the same peak.

The highest peak was reached with the sample in which complementary DNA was used as the Template for the ligation.

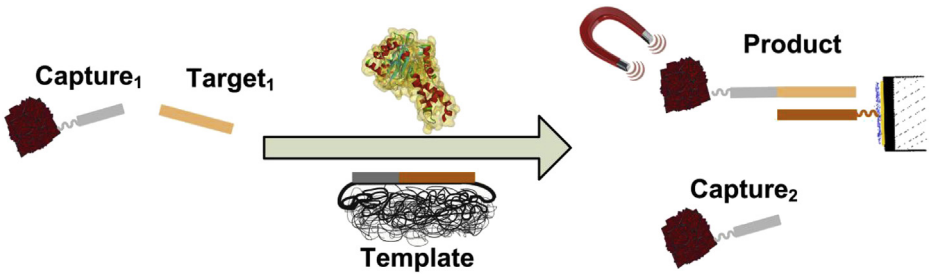

Fig. 6. Schematic diagram of the proposed detection system when different templates are tested. The concentration of Target probe is chosen as limiting factor therefore the product represents the Target probe in electrochemical study.

Surprisingly, the ligation where the Template contained a single mismatch resulted in a higher peak than with extracted genomic DNA. This might have happened due to the low concentration of the Template sequence in the genomic DNA that it was double stranded and might have needed an additional stage in the reaction. Using a larger amount of genomic DNA as a Template can also reduce the concentration of the enzyme and other important materials in the ligation reaction. The lower peak of the ligation product of mismatched Template relative to the complementary one is consistent with what has been previously discovered ie that T4 DNA ligase is capable of catalyzing the reaction but with a lower rate [36]. Increase in current in selectivity experiment was directly related to the increase in concentration of the redox complex on the surface of the electrode due to the higher interaction with hybridized analyte compared to single stranded DNA probe.

3.5. Sensitivity of the system

In the experiment for testing the sensitivity of the system, complementary Template DNA was used in different concentrations. Here, as explained earlier in Section 3.4, the limiting factor of the ligation reaction was set to be the Template. A concentration ten times higher than that of the Target was used in the knowledge that the maximum that could remain at the end of the separation stage would be equal to the concentration of the Template if the process was completely successful.

In the proposed detection mechanism the final product of the ligation reaction remains in the sample after the magnetic separation. To be able to differentiate different concentrations of the complementary Template DNA, we chose the concentration of the complementary Template DNA as the limiting factor (Fig. 7). Thus, by adjusting the initial concentrations:

$$\text{Capture}_1 > \text{Target}_1 > \text{Template}$$

The concentration of the Template DNA was the least of the components in the following reaction:

$$\text{Capture}_1 + \text{Target}_1 \rightarrow \text{Capture}_2 + \text{Target}_2 + \text{Product}$$

At the end of the reaction, the concentration of the Template DNA remains constant, the amount of the Target probe equal to the amount of the Template DNA becomes the ligation product and the extra Target probes and Capture probes remain untouched.

Therefore:

$$\text{Capture}_2 = \text{Capture}_1 - \text{Template}$$

and

$$\text{Target}_2 = \text{Target}_1 - \text{Template}$$

The concentration of the ligation product is equal to the concentration of the limiting factor which in this case is the Template DNA:

$$\text{Product} = \text{Template}$$

Fig. 8a shows the DPV result of the final product of the ligation in which different concentrations of the Template DNA between 1×10^{-7} M and 3.13×10^{-9} M were employed. There was a direct relationship between the concentration of the Template DNA and the achieved current, with a correlation coefficient of 0.968 (Fig. 8b). The detection limit of the system (3.3N/S) was calculated as 5.37×10^{-14} M. Although the detection of the designed Target probe with the constructed electrode had previously revealed a limit of detection of 1.59×10^{-17} M [10], the proposed new procedure was highly selective in the detection of the genomic DNA. Additional separation and washing steps in this method increase the detection limit but they are crucial in a system in which the key features are MNPs and enzyme (ligase).

4. Conclusion

Electrochemical nanosensors are important analytical tools as the demand for sensitive, rapid, and selective detection of analytes increases in the fields of healthcare, environmental monitoring, and biological analysis and are already demonstrating broad application and some early success as reliable, portable devices [37].

However, researchers are still grappling with designing increased specificity and sensitivity as well as resolving a range of other issues including biocompatibility and stability [37].

More specifically, in the field of electrochemical DNA sensing, the detection of genomic DNAcontinues to be frustrating. Two of the main reasons for this are the composition of DNA itself; it is double stranded and it exists in very long chains whereas most DNA sensing probes are designed in a small number of bases that Target only a miniscule part of the DNA. And, additionally, in such electrochemical sensing, the analyte needs to be brought to the biorecognition surface.

Experimenting in these two important areas was the underpinning purpose for this research. The objective of the work described in this paper was to detect small sequences in large amounts of double stranded DNA and thus to improve the selectivity and sensitivity of a developed DNA biosensor.

Building on the earlier effort, we continued to use a modified gold electrode and gold nanoparticles but this time we attached functionalised Fe_3O_4 magnetic nanoparticles to the Capture probe. This enabled a form of indirect sensing and successfully facilitated

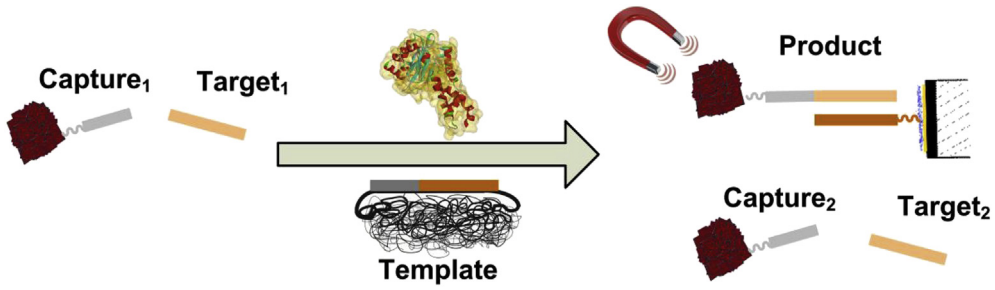

Fig. 7. Schematic diagram of the proposed detection system when different concentrations of the templates are tested. The concentration of Template is chosen as limiting factor therefore the product represents the Template in the electrochemical study.

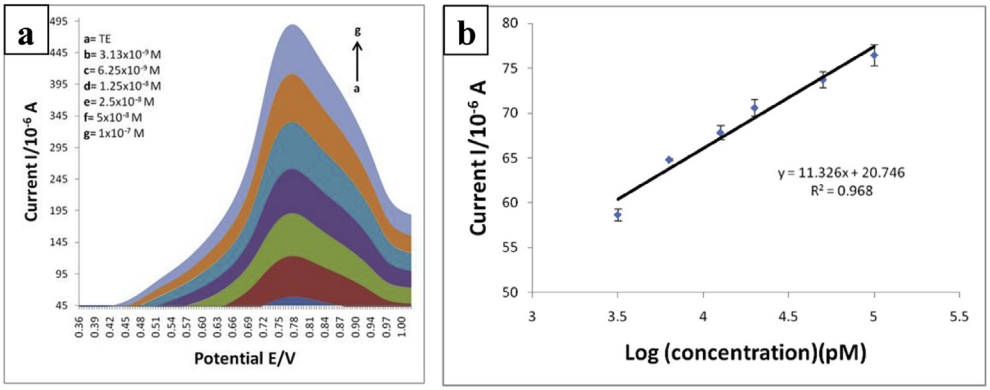

Fig. 8. a) Evaluation of the products of different concentrations of the complementary Template by differential pulse voltammetry (DPV) b) linear plot of peak current against log [concentration] of target DNA.

bringing a representative sequence of the analyte to the bio-recognition surface.

But the most novel aspect of this work came through the ligation between the Target and Capture probes mediated by the enzyme, T4 ligase which ensured ultimate detection of the Template (genomic) DNA. Although performed only once in this work, this technique has the potential to be continuously repeated in ligation chain reactions and thus the ability to detect even smaller amounts of DNA.

Whilst the challenge of detecting small Target sequences in large amounts of extracted genomic DNA is not yet over in the search for early detection of *Ganoderma*, it is hoped that the technique described here can make a positive contribution and will be quickly developed further not only in the detection of a wide range of pathogens but also any types of genomic DNA.

Conflict of interest

The authors have no conflict of interest to declare.

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